Supplementary electronic information

Solubilization of SWNTs by alkyl-sulphate chitosan derivatives of different molecular weight: towards the preparation of hybrids with anticoagulant properties

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Materials and methods

Synthesis of *N*-octyl chitosan

Chitosan (1.0 g) was suspended in methanol (50 mL) and octaldehyde (1.02 g) was added to the suspension while stirring; the suspension obtained was stirred at room temperature for 24 hours. An aqueous solution of NaBH4 (0.5g in 5 mL) was slowly added to the reaction mixture and the resulting mixture was stirred at room temperature for further 24 hours. The reaction was stopped by neutralization with 2 M HCl. The product was filtered and repeatedly washed with methanol and water and finally dried under vacuum at 60°C to constant weight.

***N*-octyl-*O*-sulphate chitosan**

N-octyl-chitosan (1.0 g) was suspended in DMF (40 mL). Chlorosulfonic acid (20 mL) was added dropwise to 40 mL of DMF and the mixture stirred for 1 h at 0 °C. Then the *N*-octyl-chitosan suspension was added to the above solution. The mixture was reacted at 40 °C for 24 hours. The reaction was stopped by neutralization with 20 % w/v NaOH, the obtained precipitate was filtered off and the filtrate was dialysed against distilled water for 3 days and then freeze dried.

Purification and characterisation of SWNTs

Pristine nanotubes were purified using two different methods, namely: microwave assisted purification[1] and purification by oxidation in nitric acid.[2]

Microwave purification: Microwave assisted purification of SWNTs was carried out as described in literature with a slight modification. Carbon nanotubes (20.0 mg) were placed into a 100 mL conical flask and into a conventional microwave at 90 W for 5 s; the carbonaceous material was removed from the microwave, shaken and stirred with a spatula, and placed back into the microwave for another 5 s (x 60) of treatment. The SWNTs were then suspended in concentrated HCl (10 mL), the mixture obtained was centrifuged at 4,000 rpm for 5 min. After discarding the supernatant the SWNTs were further washed with water (10 mL), methanol (10 mL), and diethyl ether (10 mL). The remaining solid was dried using compressed air (10.8 mg).

Nitric acid oxidation: SWNTs were oxidised by treatment in nitric acid as described in literature with modifications. HipCo SWNTs (20.0 mg) were suspended in nitric acid (4 mL) and the mixture was refluxed at 88 °C overnight. Deionised water was added to stop the reaction and the mixture was centrifuged at 4,000 rpm for 5 min. The SWNTs were washed twice with water and the final solution was neutralized with NaOH (20 % w / v). The modified SWNTs were separated by centrifugation (4,000 rpm, 5 min) and dried under a vacuum (19.6 mg).

Cytotoxicity assay method

Caco-2 cells were seeded in 96-well plate and incubated for 2 days in culture medium (Media 199, Invitrogen, supplemented with FCS (10 %) and penicillin / streptomycin (50 µg/mL) at 37 °C in air (95 %) : CO2 (5 %). Media was removed and cells were incubated for 2 h with polymer (500 µL) dissolved in Media 199, at concentrations of 0.1, 1 and 10 mg/mL. SDS (10 mg/mL) was used as positive control and culture media as reference for 100% cell viability. Media and test solutions were then removed after treatment time and cells washed with PBS. Freshly prepared MTT (200 µL) in Media 199 (0.5 mg/mL), without any additions, was added and cells were incubated for 1 h at 37 °C. Subsequently, MTT solutions were removed from the wells and DMSO (400 µL) was added to dissolve formed formazan crystals. After homogeneous agitation (10 min), the absorbance was read at 584 nm.

In vivo biomodifcation of NOSC-SWNTs dispersions

Brine Shrimp (Artemia) cysts (NT Labs, United Kingdom) were incubated in artificial sea water (Tropic Marin, Wartenberg, Germany) at 27 °C for 24 h with constant lighting until hatching. A small number of resulting larvae (about 20 -25 individuals in triplicates) were incubated for 24 h at 27 °C as reported previously3 in a 48 well-plate with 20 µL of PBS and 980 µL of NOSC-SWNTs suspensions. Artemia larvae were counted and photographed with the use of a stereoscope (GX Microscopes) and a digital camera (LEICA IM50).

Results and discussion

Sulphation of NOC, mechanism of reaction

The second step of the reaction involved the sulphation of NOC in chlorsulfonic acid and DMF. The level of solvation of the polymer at the beginning of the reaction is of paramount importance for the successful sulphation of chitosan as this determines the reactivity of the polymer.[4] For this reason NOC was initially swollen in DMF. Chlorsulfonic acid reacts with DMF by O-acylation, forming salt (I) which is the actual sulphating agent (Figure 1). Salt (I) can then react with a nucleophilic group such as primary amines or primary or secondary alcohols on the polysaccharide backbone with release of DMF.[5]

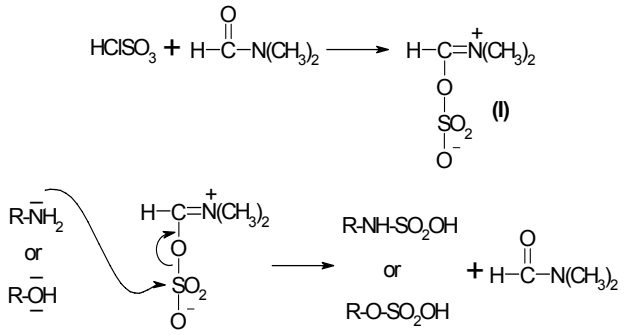


Figure 1. Proposed mechanism of sulphation using the sulphating mixture chlorsulfonic acid and DMF.

The successful synthesis of NOSC was confirmed by ATR analysis; alkylation of chitosan in position C2 is shown in Figure 2 (iii) by the disappearance of the peaks assigned to the primary amino group of chitosan (-NH_2 bending vibrations at 1650 and 1590 cm^{-1}), whilst new peaks attributed to the alkyl chain were identified at 2961 and 2874 cm^{-1} (Figure 2 ii).[6-7]

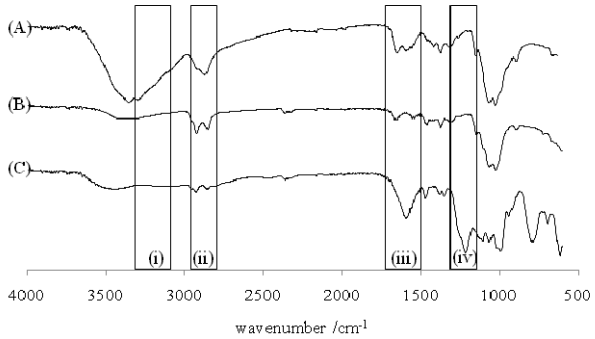


Figure 2. ATR spectra of (A) chitosan (low molecular weight), (B) *N*-octyl chitosan and (C) *N*-octyl-*O*-sulphate chitosan, respectively.

Figure 2 (i) shows that the introduction of the octyl group has an effect on the intermolecular hydrogen bonding of the polysaccharide, in fact, after modification, the two peaks in the region of 3100-3300 cm^{-1}, assigned to the N-H and O-H stretching restricted by the hydrogen bonding, disappear.[8] ATR analysis also revealed that *O*-sulphation occurred mainly in position C6 as the peak attributed to the combination of O-H bending and C-O stretching of the primary alcohol (1150 cm^{-1}) disappeared and new peaks assigned to O=S=O and C-O-S appeared at 1249, 1211, 995, 805 cm^{-1} (Figure 2 (iv)).[4,9-10,11]

Alkylation was also visible on the 1H-NMR spectrum in which new peaks at 0.8-1.0 ppm (-NH-CH2-(CH2)6-CH3), 1.2-1.6 and 1.7-1.8 ppm (-NH-CH2-(CH2)6-CH3) and 3.4-3.5 ppm (-NH-CH2-(CH2)6-CH3) were observed (Table 1). Sulphation in position C6 was also confirmed by the presence of two broad peaks between 3.7 and 4.1 ppm of the 1H-NMR spectrum, assigned to the protons of the modified C6.[12] Both from elemental analysis data and ATR data (i.e. decrease of intensity of the peaks at 2961 and 2874 cm^{-1}) it can be observed that the sulphation reaction causes partial loss of the alkyl chain in position C2, this is in accordance with previous reports.[13] The data reported in Table 1 also show that the sulphation degree obtained deceases with the increase in the molecular weight of the NOC polymer employed in the reaction; this could be due to a lower solubility and chain flexibility of higher molecular weight polymers. The effect of alkylation and sulphation on the crystal structure of chitosan were studied by XRD and are in agreement with our previous report.[14]

Table 1. NMR spectra analysis for NOSC.

Chemical shift (ppm)	Assignment
0.8-1.0	-NHCH$_2$(CH$_2$)$_6$<u>CH$_3$</u>
1.2-1.6 1.7-1.8	-NHCH$_2$(<u>CH$_2$</u>)$_6$CH$_3$
2.0-2.1	-NH-CO-<u>CH$_3$</u>
3.1-3.5	2H in C2 (overlapping with next peak)
3.4-3.5	-NH<u>CH$_2$</u>(CH$_2$)$_6$CH$_3$
3.7-4.1	2H in substituted C6
4.2-4.7	HOD and all other H
4.7-4.8	1H in C1

Thermal analysis of the polymers

Further characterisation was performed by means of TGA studies that revealed that the polymers tested exhibit an initial weight loss at temperatures below 100 °C, this corresponds to loss of water adsorbed onto the polymer, due to the fact that polymers did not undergo a process of drying before analysis (Fig. 3). All polymers presented a degradation temperature in the range of 200 – 300 °C (Table 2). This temperature decreased with the decrease in crystallinity observed when the alkylation (NOC) and successive sulphation (NOSC) of chitosan were carried out and indicated that all derivatives had lower thermal stability compared to chitosan,[15] probably due to the decreased intra- and inter-chain H-bonding capacity of the derivatives.[16] Another factor that was affected by the chemical modification of chitosan was the weight loss registered during the degradation process which was highest in every case for the alkyl derivative (NOC) and lowest for the NOSC derivatives.

Table 2: Degradation temperature and weight loss during thermal degradation of chitosan and its derivatives.

Polymer	Thermal degradation in N2	
	DTG max (°C)	%wt loss
L-chitosan	291.92	55.93
L-NOC	285.73	78.35
L-NOSC	238.38	55.02
M-chitosan	292.53	48.94
M-NOC	273.13	69.46
M-NOSC	238.07	38.07
H-chitosan	295.46	53.20
H-NOC	283.51	66.64
H-NOSC	209.40	36.46

A relationship between molecular weight and degradation temperature was noted only for the starting chitosan; longer chains showed higher thermal stability as previously reported in literature.[17] The degradation temperature for NOC and NOSC derivatives did not show a clear correlation with the molecular weight however they showed an increase in residual mass with increasing molecular weight. The degree of modification of chitosan had some effect on the weight loss during the degradation step, with higher weight loss observed as the degree of sulphation increased and the degree of alkylation decreased.

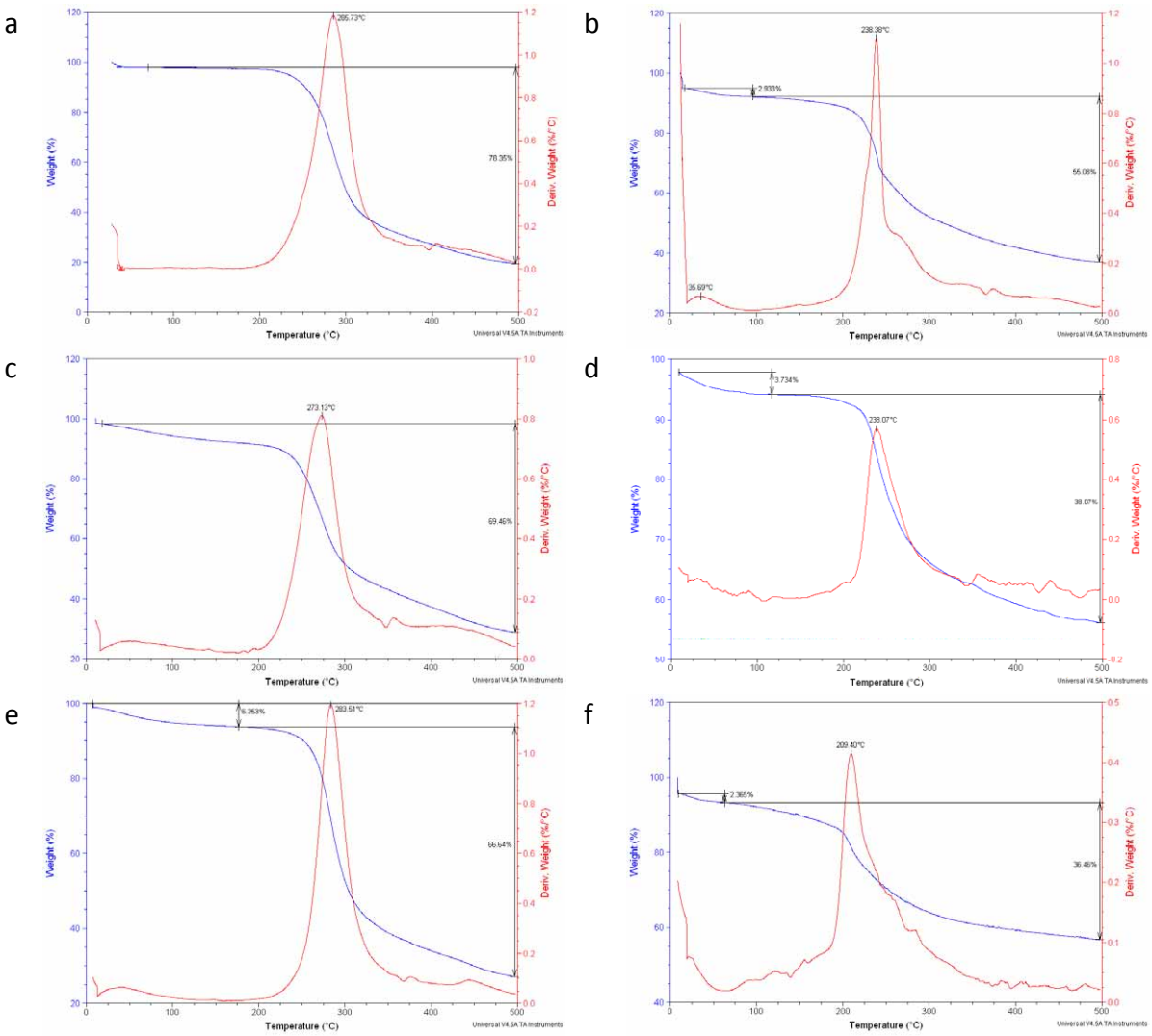


Fig. 3. Thermogravimentric analysis of a) L-NOC, b) L-NOSC, c) M-NOC, d) M-NOSC, e) H-NOC and f) H-NOSC.

Raman studies: Tangential Modes (G-band)

The spectral envelope 1400–1650 cm^{-1} is dominated by two main bands, i.e. high G^+ and low G^- frequency bands that correspond to respective vibrations along the tube axis and along the circumferential direction of SWCNTs.

These bands provide a means for the assessment of the metallic character of such materials[18]. The G^- band of semiconducting nanotubes exhibits a Lorentzian profile whereas that of metallic nanotubes has an asymmetric profile which is adequately described using Breit-Wigner-Fano (BWF) line-shape analysis[7]. The frequency and the width of the asymmetric G^- band, described by BWF, depend on the strength of the inter-tube interaction. Accordingly, Fig. 4 shows the tangential mode region of SWCNTs in the pristine Figure 4a and microwave purified 4b form, and in NOSC dispersions 0.5 mg ml^{-1}. The feature at ~1335 cm^{-1} is attributed to the D-band (disorder–induced scattering). The G^+ band of SWCNTs in dispersions is narrower (and slightly blue-shifted) than that of pristine nanotubes. Normalisation of the spectra at the G^+ band maximum reveals a severe suppression of the intensity of the G^- band of SWCNTs from dispersion relative to that of the powder. However, there is no complete vanishing of the BWF asymmetric lineshape in dispersions. This result suggests that the origin of the BWF lineshape, i.e. the coupling of a discrete energy excitation level to a continuum of phononic or electronic excitations, is an intrinsic feature of the SWCNTs. The existence of small, sharp peaks in the spectra, on top of the asymmetric G^- band, is possibly associated with the presence of semiconducting SWCNTs in the sample.

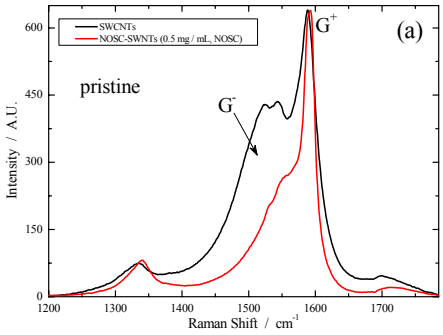


Figure 4a. Stokes-side Raman spectra of the tangential modes (G–band) and (G– band) regions respectively profiles for pristine and NOSC-SWCNTs dispersions 0.5 mg / mL.

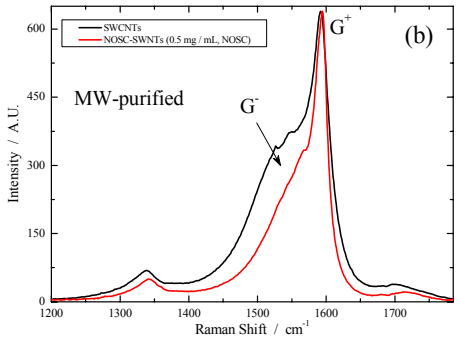
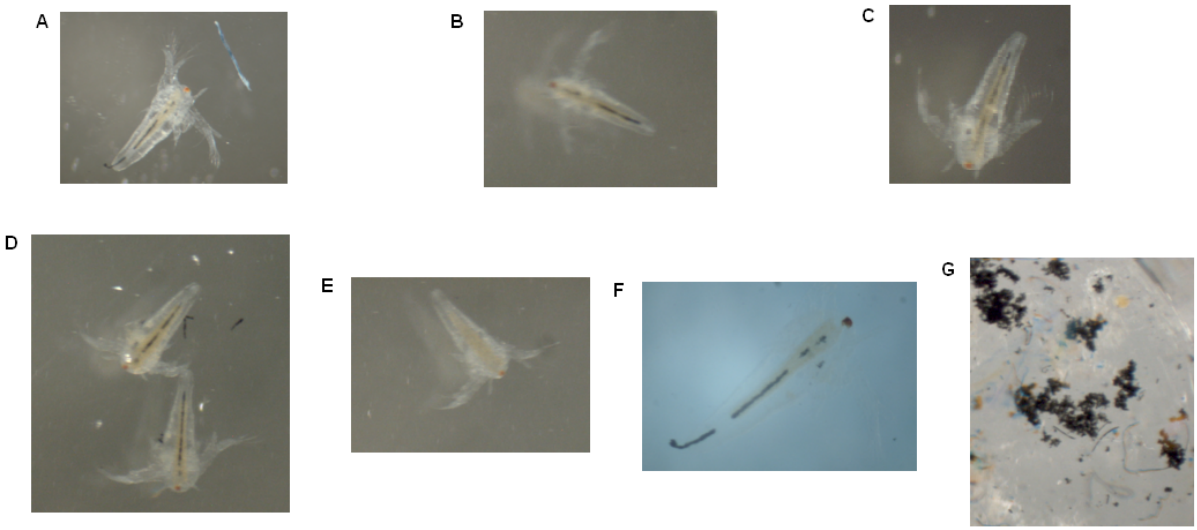


Figure 4b. Stokes-side Raman spectra of the tangential modes (*G*–band) and (*G*⁻ band) regions respectively profiles for microwave purified SWNTs and NOSC-SWCNTs dispersions 0.5 mg / mL.

Interactions of NOSC-SWNTs dispersions with Artemia

Preliminary studies on the toxicity of NOSC coated SWNTs were performed in an aquatic model organism (Artemia) which is a well established system for the assessment of ecotoxicity and cytotoxicity.[3, 19] Exposure to NOSC-SWNTs suspensions with variable NOSC concentrations (0.1 - 0.3 mg / mL), microwave purified SWNTs alone (0.1 mg / mL) and NOSC alone (0.3 mg / mL) exhibited no effect on the viability of sub-adult Artemia. The tolerance of Artemia larvae to undiluted and suspended SWNT at 24 h after hatching incubation is verified with the observation that nanotubes accumulated in the animal irrespectively of the amount of polymer present in the hybrids (Fig 5 A-F). However, NOSC-SWNT suspensions are disrupted Artemia larvae partially due to movement and feeding action of the Artemia larvae over 24 h (Figure 5 G). Nevertheless, the portion that is passed through the organism due to filter feeding with NOSC-SWNTs is in vivo biomodified and excreted in the form of enveloped dark rod-like structures (Figure 5 F). This permissive behaviour of Artemia larvae towards NOSC-SWNTs recorded in this preliminary study, hints at the possibility that NOSC-SWNTs may



be used as biocompatible and/or biomodifiable building components for biomaterials.

Fig. 5. Artemia exposure to L-NOSC-SWNTs; A. L-NOSC-SWNTs (0.1 mg / mL L-NOSC); B. L-NOSC-SWNTs (0.2 mg / mL L-NOSC); C. L-NOSC-SWNTs (0.3 mg / mL L-NOSC). D. Microwave purified SWNT (0.1 mg / mL) E. L-NOSC (0.3 mg / mL) F. Bright field visualization of nanotube accumulation in Artemia. G. In *vivo* biomodification of NOSC-SWNTs suspensions. Magnifications were for A-E X300 and for F X600.

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